EXHIBIT D

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A Novel Form of Tissue-Specific RNA Processing Produces Apolipoprotein-B48 in Intestine

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Summary

Evidence suggests that intestinal apo-B48 is colinear with the amino-terminal half of hepatic apo-B100. To investigate the mechanism of apo-B48 production, we examined cDNA clones from human and rabbit small intestine. All clones contained a single C → T base difference from the hepatic sequence, resulting in a translational stop at codon 2153. Amplification by the polymerase chain reaction of cDNA from human and rabbit small intestine, rabbit liver, and the human hepatoma cell line HepG2 showed that the stop codon was only present in intestinal mRNA. Enterocyte genomic DNA did not contain the stop codon. We suggest that a co- or posttranscriptional C → U change may result in the production of apo-B48, which represents the amino-terminal 2152 amino acids of apo-B100. This is the first example of tissue-specific modification of a single mRNA nucleotide resulting in two different proteins from the same primary transcript.

Introduction

Two closely related forms of apolipoprotein (apo-) B circulate as obligatory constituents of the plasma lipoproteins (Kane, 1983). In humans, apo-B100 (M, 512 kd) is synthesized in the liver and is necessary for the assembly of very low density lipoproteins (VLDL) and for the transport of endogenously synthesized triglyceride. It is also the ligand that mediates the clearance of low density lipoprotein (LDL) cholesterol from the circulation by the LDL receptor pathway (Brown and Goldstein, 1986). The other form of apo-B is synthesized by the intestine and designated apo-B48 on the centile system because it is roughly half the size of apo-B100. It is essential for chylomicron formation and the absorption and transport of dietary cholesterol and triglyceride.

The complete cDNA sequence of apo-B100 (Knott et al., 1986a; Cladaras et al., 1986; Law et al., 1986; Yang et al., 1986; Olofsson et al., 1986) and intron–exon organization of the apo-B gene have been deduced (Blackhart et al., 1986; Carlsson et al., 1986; Higuchi et al., 1987; Wagener et al., 1987). Human apo-B100 mRNA is 14121 nucleotides in length and encodes a 4563 amino acid precursor from which a 27 residue signal peptide is cleaved. The corresponding gene spans only 43 kilobases (kb) and is comprised of 29 exons. Over half of the coding sequence is carried by the 7572 bp exon 26.

Antibody and peptide mapping, protein sequencing, and amino acid composition studies suggest that apo-B48 represents the amino-terminal half of apo-B100 (Hardman and Kane, 1986; Marcel et al., 1987; Olofsson et al., 1987). Several lines of evidence predict the region of apo-B100 in which the apo-B48 molecule is likely to end. Epitopes for monoclonal antibodies against apo-B have been mapped on apo-B100/β-galactosidase fusion proteins and proteolytic fragments of apo-B100 (Knott et al., 1986a; Marcel et al., 1987). All monoclonal antibodies mapping to the amino-terminal half of apo-B100 also react with apo-B48. A further group do not recognize apo-B48 (Knott et al., 1986a; Marcel et al., 1987) and have been localized to the carboxy-terminal half of apo-B100. There are no apo-B48-specific monoclonal antibodies. Furthermore, apo-B48 does not interact with the LDL receptor (Hui et al., 1984) and the LDL receptor-binding domain of apo-B100 resides in the carboxy-terminal one-third of the protein (Knott et al., 1986a; Marcel et al., 1987). These results therefore point to apo-B48 being colinear with the amino terminal half of apo-B100 and imply that it will terminate between residues 1700 and 2600. This is consistent with the observed size of apo-B48 on SDS-polyacrylamide gels (SDS-PAGE), assuming that it has a similar glycosylation pattern to apo-B100 (Knott et al., 1986a; Yang et al., 1986). In addition, the amino acid composition of apo-B48 is similar to that of apo-B100 between residues 1 and approximately 2200 (Hardman and Kane, 1986). Therefore, the carboxy-terminal of apo-B48 is likely to be within exon 25 (7572 bp) of the apo-B gene.

There are three likely mechanisms to explain the origin of apo-B48. First, apo-B100 and apo-B48 could be encoded by different genes. This has been largely excluded by the analysis of the gene structure (Blackhart et al., 1986; Carlsson et al., 1986; Higuchi et al., 1987; Wagener et al., 1987). Further strong evidence that both apo-B proteins are products of a single gene is provided by the work of Young et al. (1986), who demonstrated that the same protein polymorphism was present in both apo-B100 and apo-B48 in 23 subjects examined. Second, apo-B48 may be produced by cotranslational or posttranslational proteolytic processing of apo-B100. In support of this, monoclonal antibodies have been used to demonstrate apo-B100-specific epitopes in human intestinal cells. Also, a large form of apo-B is reported to be secreted into intestinal lymph and processed to a protein similar in size to apo-B48 in rat (Lee et al., 1984; Dullaart et al., 1986). However, pulse–chase and polysome run-off translation experi-

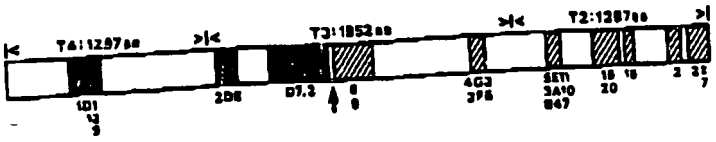

Figure 1. Epitope Map of Monoclonal Antibodies Binding to Apo-B

Solid boxes denote epitopes for antibodies that bind to both apo-B100 and apo-B48. Hatched boxes are epitopes for monoclonal antibodies that are specific for apo-B100 (Knott et al., unpublished data; Knott et al., 1986a; Marcel et al., 1987). The predicted end of apo-B48 is indicated by the arrow. The sizes of the thrombin cleavage products T4, T3, and T2 of apo-B100 are shown (Cardin et al., 1984).

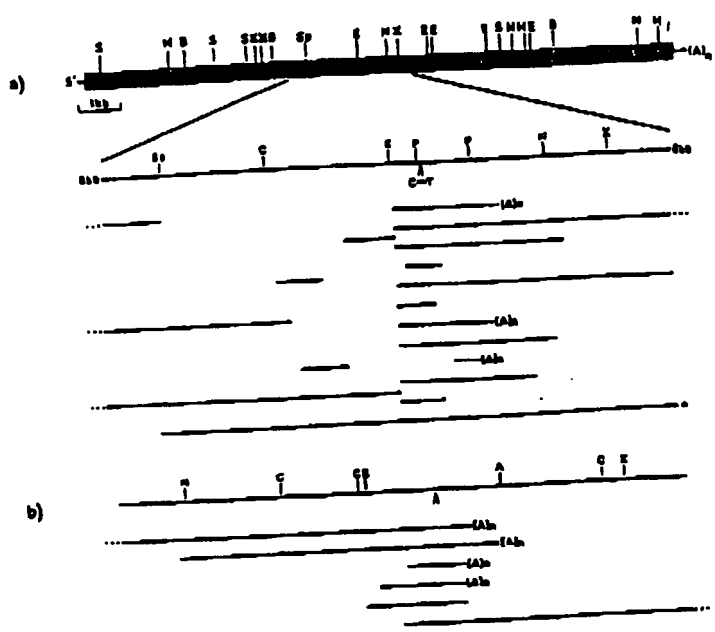

Figure 2. Apolipoprotein Intestinal cDNA Clones

(a) Restriction map of apo-B cDNA. Abbreviations: A, Acc I; Sa, Sal I; H, HindIII; B, BamHI; X, XbaI; S, SalI; E, EcoRI; C, ClaI; P, PstI. The region between 5 and 8 kb is expanded. The position of the C to T change resulting in the introduction of the in-frame stop codon is marked with an arrow. The cDNA clones that were isolated, mapped, and sequenced from the first human jejunal library are shown, including the three with poly (A) tails. The single large clone from the second small intestinal library is marked with an asterisk.

(b) Restriction map of rabbit small intestinal cDNA clones. The position of the stop codon is indicated by the arrow.

ments provide no evidence of proteolytic processing (Reuben and Elovson, 1965; Demmer et al., 1986; Glickman et al., 1986). Third, differential processing of primary nuclear RNA transcript could produce distinct mRNAs coding for apo-B100 and apo-B48 in the liver and intestine, respectively. Several groups have reported Northern blots showing two apo-B mRNAs in the small intestine, an abundant 14.5 kb mRNA and an mRNA of approximately 7.0 kb that hybridizes with 5' but not 3' apo-B100 cDNA probes (Deeb et al., 1985; Mehrabian et al., 1985; Cladaras et al., 1986; Olofsson et al., 1987). However, the detection of the small transcript has been inconsistent and this has led to the suggestion that it may be a degradation product of the larger mRNA (Mehrabian et al., 1985).

The aim of the present study was to investigate the molecular basis for the production of apo-B48 by examining clones from human and rabbit small intestinal cDNA libraries and comparing these with the sequences of hepatic cDNAs and genomic DNA.

Results

Apo-B cDNA from Human and Rabbit Small Intestine Shows a C to T Substitution at Nucleotide 6666

We have focused on the region between 5 and 8 kb from the 5' end of the mRNA that was delineated by the mapping of monoclonal antibodies as shown in Figure 1.

Clones covering the entire 14.1 kb coding region were isolated from a human mid-jejunal cDNA library using probes from the whole length of apo-B100 cDNA. Detailed restriction mapping of cDNA clones failed to demonstrate any differences, aside from previously identified common polymorphisms (Blackhart et al., 1986), between the hepatic (Knott et al., 1986b) and intestinal mRNA except in the region where we predict apo-B48 to end. In all ten intestinal cDNA clones (Figure 2a), which included nucleotide 6666 of the apo-B100 mRNA sequence, an in-frame stop codon (TAA) was found after codon 2152. Amino acid 2153 is glutamine (CAA) in all five published apo-B100 he-

Table 1. Positions of Potential Polyadenylation Signals

	Sequence		Position of Poly (A) Tail
Human Clone			
Hu 1	ACCAAA	9 bp	6910
Hu 2	AATAAA	19 bp	7080
Hu 3	TATAAA	24 bp	7121
Rabbit Clone			
Rab 1 + 2	AATTAA	17 bp	6774
Rab 3	AATTAA	15 bp	6815
Rab 4	AATAAA	19 bp	7080

The positions of the potential polyadenylation signals relative to the sites of polyadenylation in the human and rabbit cDNA clones are shown. There are three different hexamers that may act as polyadenylation signals in addition to the canonical AATAAA. One of these, AATTAA, has been previously recognized as a functional hexamer (Birnstiel et al., 1985). The other two (Hu 1 and Hu 3) have not been previously implicated in polyadenylation. However, the only other sequences 5' to these sites that could be used as signals for polyadenylations are AACAAA (61 bp 5' of the site used in Hu 1) and AATAAA (60 bp 5' of Hu 3). Polyadenylation can occur 42 bp downstream of a polyadenylation signal (Kessler et al., 1987).

patic cDNA sequences and in all three published genomic sequences.

In addition, three of the human intestinal cDNA clones from this region were polyadenylated at different positions downstream of the termination codon (Table 1). This confirms the presence of a 7 kb apo-B mRNA species in human intestine. The remaining cDNA clones were not polyadenylated, and contained sequences extending up to 1.4 kb downstream of the stop codon (Figure 2a).

To eliminate the possibility of cloning artifacts or of mutation in this subject, we examined clones from a different human jejunal library and from a rabbit small intestinal library (Hunziker et al., 1986); the rabbit is also known to produce both the 14.5 kb and the 7 kb apo-B mRNAs (Cladaras et al., 1986). The stop codon was present at the same position in the only cDNA clone isolated from the second human intestinal library that carried sequences in the region of interest (Figure 2a). In the rabbit, all six small intestinal cDNA clones (Figure 2b) from the comparable region also contained a stop codon (TAA) replacing the normal glutamine codon (CAA) at the identical position. Poly (A) tails were also found in the rabbit clones, but the sites of polyadenylation were different from those occurring in the human intestine (Table 1). A comparison of the rabbit sequence to that of the human (Figure 3) shows 85.8% homology between nucleotides 5000–6000 and 78.7% homology at the protein level. The DNA sequence conservation increases to 90% between nucleotides 6586–6766 spanning the stop codon.

There Are Two Species of Apo-B mRNA in the Intestine of Human and Rabbit

Northern blot analysis of intestinal mRNA from both rabbit and human tissue (Figure 4) confirms the presence of two apo-B messages. Hybridization with probes derived from the 3' half of apo-B100 detects a single species of approximately 14.5 kb, equivalent to the 14,121 base message characterized from the liver cDNA libraries (Knott et al., 1986b). However, hybridization with probes from the 5' end of apo-B100, the region encoding apo-B48, detects the large message and a smaller 7 kb species.

The 14.5 kb and 7 kb Intestinal mRNAs Both Contain a UAA Stop Codon

Because of the small size of the intestinal cDNA clones, it is not certain that those lacking a poly (A) tail derive from the 14.5 kb transcript. It is important to determine whether the 14.5 kb mRNA contains the stop codon and encodes apo-B48 or whether it encodes apo-B100. To study this, the relevant mRNA sequences were enzymatically amplified by the polymerase chain reaction (PCR) (Saiki et al., 1985) and screened with synthetic oligonucleotides designed to detect the single C to T base change.

RNA was isolated from terminal ileum from a third human subject, rabbit liver, rabbit jejunum, and the hepatoma cell line HepG2 (known to produce apo-B100). First strand cDNA synthesis was primed either with oligo (dT) or synthetic oligonucleotides PCR9 and PCR11 (see Experimental Procedures). Use of oligo (dT) and PCR9 ensures that sequences from both the 14.5 and 7.0 kb mRNAs are represented in the cDNA, whereas PCR11 directs synthesis of cDNA exclusively from the large transcript. Second strand synthesis was completed by the method of Gubler and Hoffmann (1983), and amplification performed using specific primers as described in the legend to Figure 5 and Experimental Procedures. The amplified segment (281 bp) spans nucleotides 6504–6784 of the human mRNA and a corresponding region of the rabbit message. Amplified cDNAs were run on agarose gels, blotted onto Zetaprobe, and hybridized sequentially with oligonucleotide BGLN (TACTGATCAAATTGTATCA), which is specific for DNA containing a C at position 6668, and a second oligonucleotide, BSTOP (TACTGATCAAATTATATCA), which is specific for DNA containing the stop codon. Hybridization and washing conditions for both oligonucleotides were determined empirically (Figure 5). In addition, serial 1:10 dilutions of the PCR reactions were dot-blotted onto Zetaprobe membranes (Figure 5). HepG2 and rabbit liver cDNA PCR only hybridized with oligonucleotide BGLN. Rabbit small intestine cDNA PCR hybridized strongly with BSTOP oligonucleotide and only weakly with BGLN oligonucleotide. Human terminal ileum cDNA PCR hybridized strongly with BSTOP and very weakly with BGLN.

These observations indicate that both the 14.5 kb and 7.0 kb mRNAs from human ileum produce mainly apo-B48 and very little, if any, apo-B100. The same is true of rabbit jejunum. No transcripts containing the stop codon can be detected using this method in the RNA from rabbit liver or the human hepatoma cell line HepG2.

Apo-B Is a Single Copy Gene

One explanation for these results is that the apo-B gene is duplicated such that one gene copy carrying the CAA sequence is transcribed in the liver and the second copy

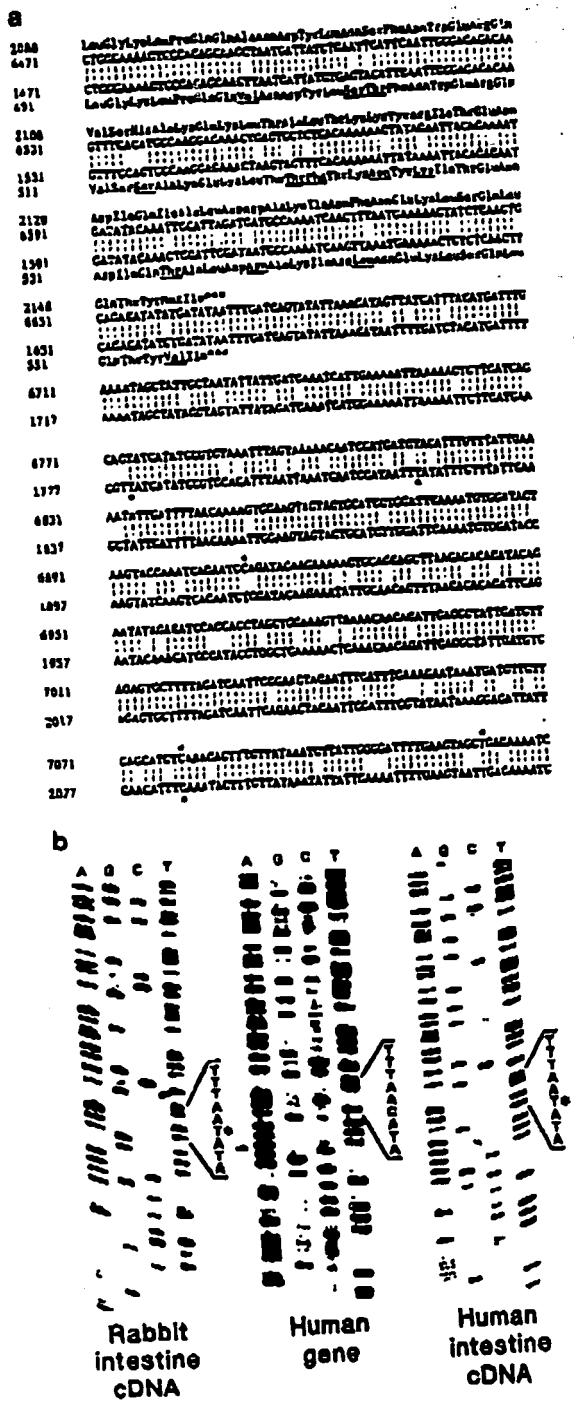

Figure 5. Nucleotide Protein Sequence of Human and Rabbit Genomic DNA

(a) Comparison of the DNA and protein sequence from the human and rabbit small intestine cDNA. The human nucleotide sequence starts at position 6741 (amino acid 2068). Three kilobase pairs of rabbit sequence has been determined (data not shown). The numbering of the homologous rabbit sequence starts at 1; the displayed sequence begins at 491. Amino acid differences are underlined for the rabbit. The positions at which poly (A) tails were added are marked (*); also see Table 1.

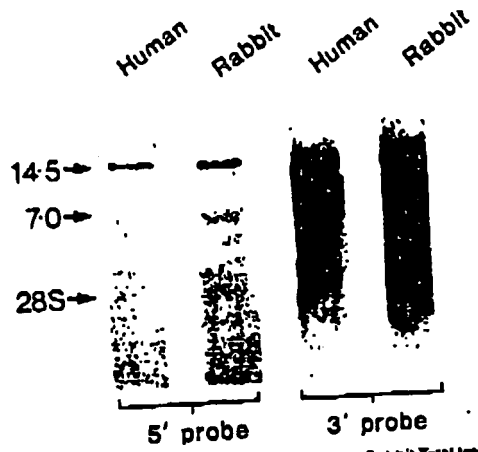

Figure 4. Northern Blot Analysis of Human and Rabbit Total Intestinal RNA

Lanes 1 (human) and 2 (rabbit) were hybridized with a probe consisting of the 5' 520 bp of apo-B100 cDNA. Lanes 3 (human) and 4 (rabbit) were hybridized with a 2 kb fragment comprising the 3' end of the apo-B100 cDNA. The 14.5 kb and 7 kb intestinal apo-B mRNAs and the position of 28S ribosomal RNA are arrowed.

with the TAA sequence is transcribed in the small intestine. To examine the possibility that there may be two apo-B genes, Southern blots of human genomic DNA were hybridized with a range of flanking and internal gene probes (Figure 6). Each digest gives hybridizing fragments that are unique. Probes that detect the hypervariable region 3' to the apo-B gene (Knott et al., 1986c) reveal only two alleles in each individual. The conclusion must be that either there is a very large duplication unit (> 50 kb) undergoing extensive gene conversion, or there is only one apo-B gene.

Differential Splicing Is Not Involved in the Production of Apo-B48

An alternative explanation for the appearance of a stop codon in intestinal message is duplication of part or the whole of exon 26 coupled with tissue-specific exon shuffling. Probes taken from each end of exon 26 and from the region containing the stop codon hybridize to unique fragments on Southern blots of human genomic DNA (Figure 6). Exon 26 has been sequenced at least five times in its entirety from genomic clones by our own group and others (unpublished results and Blackhart et al., 1986; Carlsson et al., 1986; Wagener et al., 1986). There is no evidence for duplication either of exon 26 or small segments of it that might participate in alternative or cryptic splicing. In the absence of gene duplication or exon shuffling, it is necessary to invoke either somatic mutation or RNA modification as mechanisms for the production of apo-B48.

(b) DNA sequence ladders showing the C to T substitution in rabbit small intestine cDNA, human genomic DNA, and human small intestine cDNA. The altered thymidine base is marked with an asterisk.

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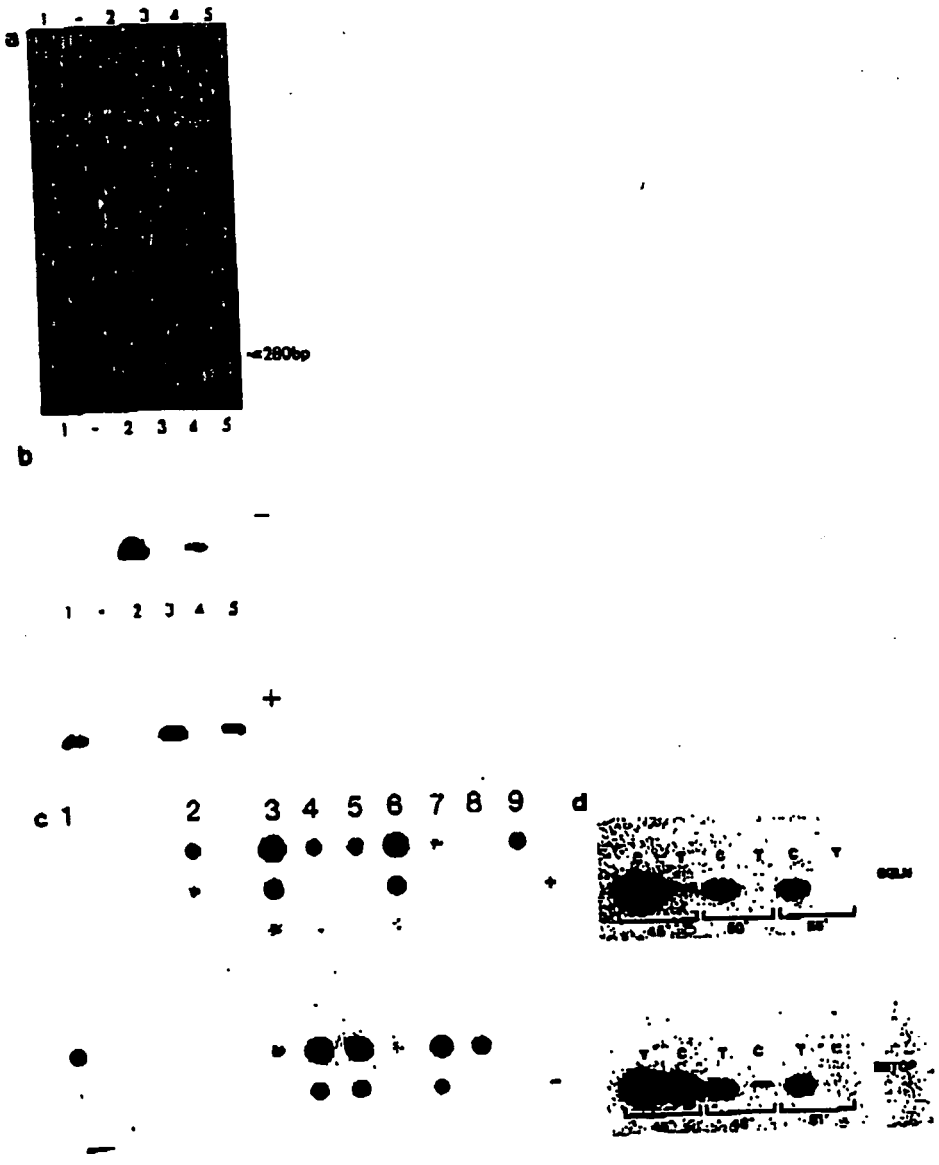

Figure 5. Differential Hybridization of Oligonucleotides to PCR Amplified cDNAs

(a) 1% agarose gel of cDNA and enterocyte genomic DNA PCR reactions. Lane 1, rabbit enterocyte amplified genomic DNA. Lane 2, rabbit small intestine PCR9 primed. Lane 3, rabbit liver total RNA PCR9 primed. Lane 4, human terminal ileum poly (A)$^+$ PCR11 primed. Lane 5, HepG2 poly (A)$^+$ oligo (dT) primed. Human cDNA was amplified with PCR5 and PCR8. Rabbit cDNA and enterocyte genomic DNA were amplified with PCR5 and PCR10. The intense ethidium staining at the bottom of the gel is due to the carrier tRNA. The specifically amplified segment is ~280 bp long. (b) Southern blot of gel (a) hybridized sequentially with BSTOP (−) and BGLN (+). Lane 1, rabbit enterocyte amplified genomic DNA. Lane 2, rabbit small intestine PCR9 primed cDNA. Lane 3, rabbit liver PCR9 primed cDNA. Lane 4, human terminal ileum PCR11 primed cDNA. Lane 5, HepG2 oligo (dT) primed cDNA. Exposure time was 12 hr with an intensifying screen at −70°C.

(c) Dot blot of PCR reactions hybridized with BGLN (+) or BSTOP (−). Lane 1, pα20C plasmid; rabbit small intestinal cDNA containing T at 6666. Lane 2, pSB9 plasmid; human genomic subclone with C at 6666. Lane 3, rabbit enterocyte genomic DNA. Lane 4, rabbit small intestine cDNA primed with PCR8. Lane 5, rabbit small intestine cDNA primed with oligo (dT). Lane 6, rabbit liver cDNA primed with PCR8. Lane 7, human terminal ileum cDNA primed with PCR8. Lane 8, human terminal ileum cDNA primed with oligo (dT). Lane 9, HepG2 cDNA primed with oligo (dT). Exposure time was 6 hr with an intensifying screen at −70°C.

(d) Discriminating washing conditions: digests of pα20C (T,STOP) and pSB9 (C,GLN) were transferred to Zetaprobe membranes and strips were hybridized with oligonucleotide BSTOP or BGLN at 42°C and washed at room temperature in 6x SSC until background was minimal. Strips were then incubated for 10 min at the temperatures shown and exposed at room temperature for 2 hr.

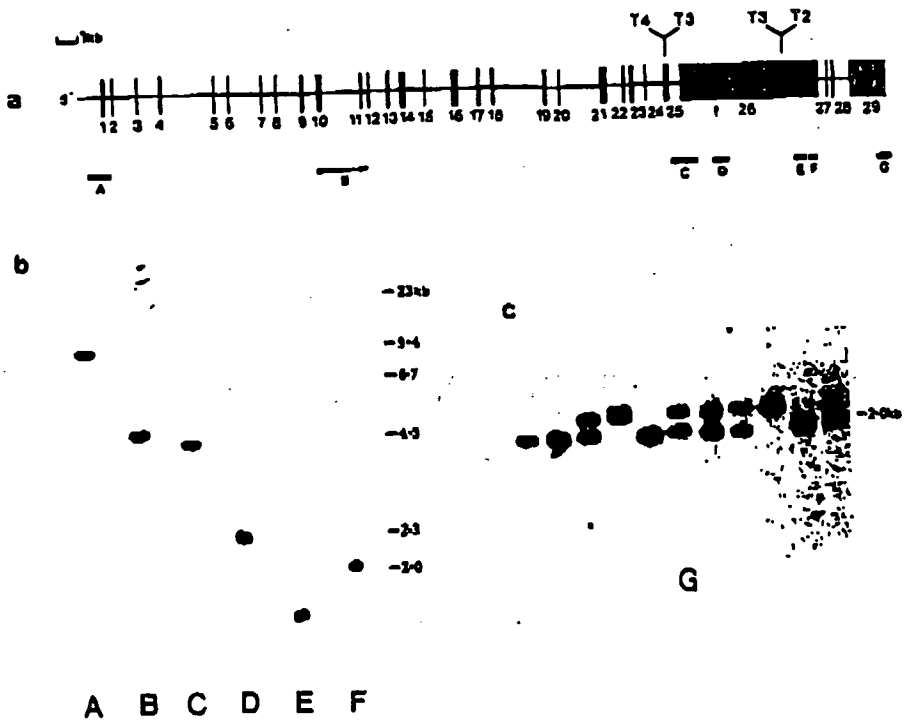

Figure 6. Southern Blot Analysis of Human Genomic DNA

(a) Intron–exon organization of the human apo-B gene. The predicted end of apo-B48 in exon 26 is indicated by the arrow. The sites of thrombin cleavage in the protein are shown. The solid bars indicate fragments used to probe genomic Southerns (b and c).

(b) and (c) Genomic Southern blots showing hybridization to unique restriction fragments flanking the apo-B gene, exon 26, and the region containing the termination codon at nucleotide 6666. Human leucocyte DNA was digested, fractionated on agarose, and transferred to nitrocellulose for hybridization with oligolabeled probes (a). Probe A is a 1.021 kb genomic fragment overlapping the 5′ end of the gene and carrying the promoter and first exon. Probe B is 600 bp BglII cDNA fragment spanning exons 10, 11, and 12. Probe C is a 1.8 kb fragment overlapping the junction between exons 25 and 26. Probe D is a 715 bp cDNA fragment that contains the region surrounding nucleotide 6666. Probes E (485 bp) and F (684 bp) are cDNA fragments from the 3′ end of exon 26 and probe G is a 350 bp genomic fragment containing the 3′ untranslated region and some flanking sequence that detects two alleles of the 3′ hypervariable region. The length polymorphisms in the hypervariable region of 11 different individuals are shown (c). The size differences are generally within plus or minus 200 bp. All lanes are EcoRI digests except B (PvuII) and G (HindIII).

The Stop Codon Is Not Present in the Genome

To investigate the possibility of somatic mutation of the apo-B gene in intestinal tissue, we carried out localized amplification of a 280 bp region spanning the stop codon using as template rabbit enterocyte DNA. Enterocytes were prepared by the method of Peters and Shio (1976) and their purity assessed by electron microscopy (Figure 7a). At least 70% of the cell population had brush borders. The region was amplified using synthetic oligomers, and the reaction run on an agarose gel. The 280 bp band was isolated and, after trimming the ends with mung bean nuclease, cloned into the Bluescript vector pKS+. One hundred colonies were picked, gridded on filters, and screened with labeled oligonucleotides. Twelve hybridizing colonies were identified. Plasmid DNA was prepared from these clones, linearized with restriction enzymes (Figure 7b), and blotted. Dot blots of uncloned amplified DNA were also prepared. Differential hybridization was carried out using the synthetic oligonucleotide probes specific for either C or T at position 6666 as described previously. Only the C specific oligonucleotide hybridizes to the 12 intestinal genomic clones (Figures 7c and 7d). Further analysis of these clones by DNA sequencing confirmed the presence of only the CAA codon in enterocyte genomic DNA. When dot blots of PCR amplified enterocyte genomic DNA were hybridized with the BSTOP and BGLN oligonucleotides, the signal obtained with BGLN was at least 100-fold greater than that obtained with BSTOP (Figure 5c).

Discussion

We set out to elucidate the mechanism of formation of intestinal apo-B by comparing the nucleotide sequences of human and rabbit intestinal cDNAs and genomic DNA with human hepatic cDNA. The result is remarkable.

In human intestinal cDNA, there is an in-frame TAA triplet at codon 2153, whereas in the hepatic cDNAs and the gene codon, 2153 is glutamine (CAA). This same change is present in rabbit intestinal cDNA. This observation is confirmed by differential hybridization to amplified cDNA and genomic DNA of labeled oligonucleotides containing either TAA or CAA. There is no likelihood that the stop codon in intestinal cDNA arose either as a result of a sys-

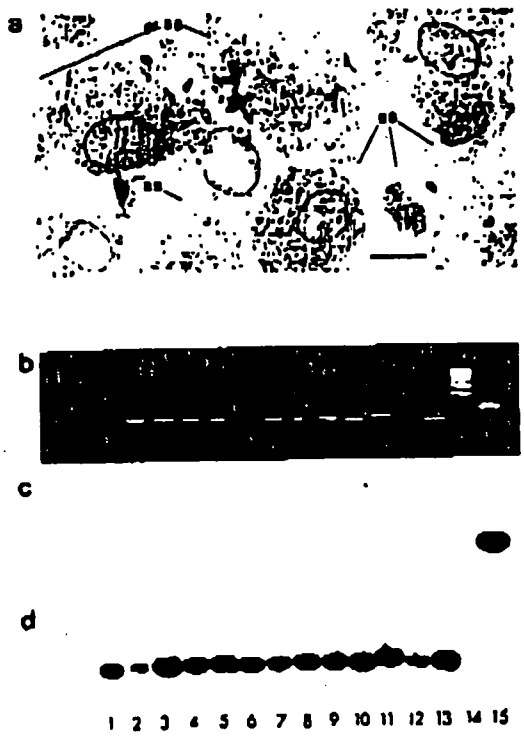

Figure 7. Differential Hybridization of Oligonucleotide PCR Amplified Rabbit Enterocyte DNA

(a) Electron micrograph of a rabbit enterocyte preparation used as the source of genomic DNA for PCR amplification. Brush borders (BB) are indicated. The scale bar represents 5 μm.

(b) Ethidium-bromide-stained agarose gel of PCR amplified, genomic DNAs cloned in Bluescript pKS+. Plasmid DNA prepared by the boiling method (Holmes and Quigley 1981) was linearized with HindIII, electrophoresed on 1% agarose, and blotted onto nitrocellulose. Lane 1, cloned, human leukocyte DNA amplified with PCR5 and PCR8. Lanes 2–13, 12 rabbit enterocyte clones. Amplification was with PCR5 and PCR9. Lane 14, markers; lambda DNA digested with HindIII. Lane 15, plasmid pa20c; a 1.7 kb rabbit intestinal cDNA carrying the stop codon cloned in pUC18.

(c) Gel (b) blotted and hybridized with oligonucleotide BSTOP and washed as described in Experimental Procedures. Only the rabbit intestinal-cDNA control plasmid with TAA hybridizes.

(d) The same filter rehybridized with oligonucleotide BGLN showing that human leukocyte and rabbit enterocyte DNA contain the CAA sequence. This was confirmed by DNA sequencing.

tematic reverse transcriptase error or as a polymerase chain reaction artifact, because CAA is found in amplified HepG2 cDNA. Our study predicts that apo-B48 is identical to the amino-terminal 2152 amino acids of apo-B100 and has a molecular weight (before glycosylation) of 240.8 kd. This is in very close agreement with the findings of Innerarity and colleagues (personal communication). These workers used antibodies and peptide mapping to predict that the carboxy-terminal of human apo-B48 would be within five residues of amino acid 2151.

The observations reported here could be explained by the presence of two apo-B genes, one of which is expressed in the small intestine and the other in the liver. If this is the case, the duplication must be perfect and encompass the whole gene and flanking sequences. Sequence analysis reveals only a few base differences between the intestinal cDNAs and our published hepatic cDNA sequence (Knott et al., 1986b), and these are consistent with polymorphic variations seen among liver cDNA clones derived from several individuals (Cladaras et al., 1985; Law et al., 1986; Yang et al., 1986; Olofsson et al., 1987). Furthermore, all genomic clones fit precisely into one linear map (Blackhart et al., 1986; Carlsson et al., 1986; Higuchi et al., 1987; Wagener et al., 1987). These observations could only be accounted for in a two-gene model if the duplication was a very recent event or if sequence homology was maintained by intragenic exchange (gene conversion). In the case of the human α-globin ($\alpha 1_1$, α_2) and γ-globin ($^A\gamma$, $^G\gamma$) genes where gene conversion events have been thoroughly analyzed (Slightom et al., 1980; Michelson et al., 1983), the various conversion units include regions of the gene that are under no apparent constraint from divergence. However, these regions of homology extend only 500 bp (γ) and 900 bp (α) upstream of the cap site and in neither case extend beyond a position equivalent to the 3′ end of the mRNA. As a consequence, the restriction enzyme sites in the distal flanking sequences vary between the globin gene duplicates. Another explanation would be the differential or cryptic splicing of an intestine-specific exon containing the stop codon. Both these possibilities have been eliminated by use of flanking sequence and intragenic probes that identify only single bands on genomic Southerns, by fine restriction mapping of cloned genomic DNA and by determining the nucleotide sequence of the gene (Ludwig et al., 1987). Specifically, there is no duplication of exon 26, which contains the stop codon, and no direct repeats that might represent alternative exons. Furthermore, enzymatic amplification of the relevant region of intestinal DNA followed by DNA sequencing and differential oligonucleotide hybridization shows that no stop codon is encoded in the rabbit genome. In the absence of gene duplication or exon shuffling, the conclusion must be that the stop codon arises as a result of co- or posttranscriptional RNA editing involving a single C to U substitution.

Our results show that both distal human and proximal rabbit small intestine contain 7 kb and 14.5 kb apo-B mRNAs. Sequencing of intestinal cDNAs from both species indicates the presence of a TAA at codon 2153 in every clone examined. It was important to establish whether all the 14.5 kb transcripts contain the stop codon and are translated into apo-B48, or whether most contained the glutamine codon and produced apo-B100. To address this issue, we amplified both specifically primed intestinal cDNA (from a position chosen to exclude all the poly (A) addition sites in Table 1 and encompass only the large mRNA) and oligo (dT) primed cDNA. We found evidence for only low levels of the glutamine codon in intestinal mRNA. The most straightforward conclusion is that both intestinal mRNAs encode apo-B48.

Previous studies from other workers have suggested that both apo-B48 and apo-B100 are produced by the adult human and rat small intestine. Dullaart et al. (1986) used monoclonal antibodies to detect apo-B100 specific epitopes in the Golgi-associated vesicles of enterocytes,

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and suggested that apo-B48 may arise by posttranslational cleavage of apo-B100. Although there were anomalies in the distribution of the apo-B100-specific epitopes which hinder interpretation of the results, this is consistent with the earlier studies of Lee et al. (1984), who reported the presence of predominantly apo-B100 in the intestinal lymph followed by rapid proteolytic conversion to a protein similar in size to apo-B48. The presence of apo-B100 epitopes in the Golgi region of enterocytes may, however, arise from endocytosis of apo-B100 containing lipoproteins, which has been demonstrated to occur in both the liver and intestine (Chao et al., 1981; Stange and Dietschy, 1983; Jones et al., 1984). Resecretion of the lipoproteins could then account for their presence in lymph, as could direct filtration from the blood into the lymph. None of these studies have proved that the apo-B100-containing lipoproteins associated with the intestine are actually synthesized there. In contrast, in vivo labeling of rat enterocytes by Davidson et al. (1986) followed by SDS-PAGE detected only apo-B48. Also, Reuben and Elovson (1985) have shown by polysome run-off translation that the product of rat intestinal apo-B mRNA is an apo-B48 equivalent protein. This supports our results showing that there is a stop codon in intestinal mRNA.

Although our data predict that adult small intestine synthesizes predominantly apo-B48, the work of Glickman et al. (1986) demonstrated that both apo-B100 and apo-B48 are produced by the human jejunum in organ cultures of tissue taken late in gestation. However, they agree that only apo-B48 is produced by this tissue in the adult. These authors performed pulse–chase studies but were not able to establish definitively that apo-B48 was produced by post-or cotranslational proteolytic cleavage.

Recent studies have shown that the colonic carcinoma cell line CaCo2 produces mainly apo-B100 and a small quantity of apo-B48 (Hughes et al., 1987). These observations suggest that intestinal cells may be pluripotent with respect to apo-B48 production until relatively late in development.

In human and rabbit intestine, a proportion of the mRNAs are polyadenylated downstream of the stop codon at sites that follow canonical and noncanonical poly (A) addition signals, whereas in human liver no small mRNA has been observed and no stop codon found. This suggests that polyadenylation and editing may be coupled.

Developmentally regulated, tissue-specific substitution of a single nucleotide in mRNA leading to different gene products is unprecedented. The closest analogy to this situation occurs in trypanosomes, where it has been demonstrated that additional nucleotides are inserted into transcripts by an RNA editing mechanism (Benne et al., 1986; Feagin et al., 1987). We cannot speculate what the mechanism might be, but it appears to be highly specific. Analogies may be found in the processing of primary nuclear transcripts and in the modified bases that are found in tRNA. Whatever the explanation for these intriguing observations, it seems unlikely that they are unique to the intestine; they may have more general biological implications.

Experimental Procedures

Construction of Human Jejunal cDNA Libraries

A section of human small intestine spanning the lower jejunum was obtained from a 15 year old female cadaver transplant donor. The tissue was cut immediately into pieces of 1–2 g, flash-frozen in liquid nitrogen, and stored at 70°C until needed. Poly (A)$^+$ RNA isolated from full thickness jejunum was used to prepare cDNA by the hairpin loop method. The cDNA was then cloned into the unique EcoRI site of the bacteriophage vector λgt11 (Huynh et al., 1985), using EcoRI linkers and transfected into E. coli Y1088. The resultant library was 90% recombinant and comprised 2 × 10^6 independent clones before amplification. The second gut cDNA library was prepared from poly(A)$^+$ RNA from a male European cadaver kidney donor using RNaseH and DNA polymerase I (Gubler and Hoffman, 1983) to synthesize the second strand.

Screening of Lambda Libraries

λgt11 phages were plated at a density of ≈25,000 per 140 mm plate on E. coli Y1088 at 42°C. Plaques were lifted on Schleicher and Schuell nitrocellulose filters and screened in duplicate with apo-B100 cDNA probes. Restriction fragments were labeled by random priming (Feinberg and Vogelstein, 1983) to a specific activity of ~$(2–10)^8$ cpm μg^{-1}. Phages were plaque-purified and DNA prepared according to the protocol of Helms et al. (1985) with the modification that the lysate from each plate was passed sequentially down two 2 ml DEAE columns and eluted from the second column. Phage insert DNA was subcloned into pUC13 for restriction mapping and into M13 mp18 or mp19 (Pharmacia/PL) for sequencing by the dideoxy method (Sanger et al., 1980). Sequence reactions were carried out with [^{35}S]dATPαS, fractionated on 6% acrylamide/50% urea gels, and visualized by autoradiography after drying (Biggin et al., 1983).

Oligonucleotides

The following oligonucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer and purified on 15% sequence gels.

BSTOP. TACTGATCAAATTATATCA 19-mer 5′ end at 6679

BGLN. TACTGATCAAATTGTATCA 19-mer 5′ end at 6679

PCR5. CTGAATTCATTCAATTGGGAGAGACAAG 28-mer 5′ end at 6504

PCR8. CGGATATATAGTGCTCATCAAGAC 25-mer 5′ end at 6784

PCR9. GCACGGATATCATAACGTTCATC 23-mer 5′ end at 6787

PCR10. CCATGATTTGATCTATAATACTAG 24-mer 5′ end at 6767

PCR11. CCAATCAGAAATGTAGGTGACAAG 24-mer 5′ end at 7767

BGLN, BSTOP, and PCR8, 9, 10, and 11 are complementary to apo-B mRNA. The coordinates above refer to human mRNA. Oligonucleotides were labeled using T4 polynucleotide kinase (Pharmacia/PL) and γ[^{32}P]ATP (Amersham PLC) as described by Maniatis et al. (1982) to a specific activity of 10^9 cpm μg^{-1}.

DNA Hybridizations

Phage DNA on nitrocellulose filters was prehybridized for >2 hr at 65°C in an excess of 5× SSPE, 10× Denhardt's (0.2% each of Ficoll, polyvinylpyrrolidone and BSA), 0.1% SDS, and 500 μg ml^{-1} denatured, sonicated salmon sperm DNA. Hybridization was in the same solution containing 2.5 × 10^5 cpm ml^{-1} [^{32}P]-labeled probe for 18 hr. Filters were washed once in 5× SSC/0.1% SDS at room temperature for 30 min, then three times in 0.1× SSC/0.1% SDS at 65°C. Zetaprobe (Bio-rad) membranes were prehybridized and hybridized to oligonucleotides in 6× SSC/0.1% SDS, 0.5% Marvel (low-fat dried milk), and 200 μg ml^{-1} denatured, sonicated salmon sperm DNA. For the BSTOP oligonucleotide, hybridization was at 42°C and washing in 6× SSC/0.1% SDS was at 51°C. For the BGLN oligonucleotide, hybridization was at 42°C and washing at 55°C.

Enterocyte Preparation

Twenty centimeters of jejunum and proximal ileum were isolated from an anaesthetized male New Zealand white rabbit (≈2 kg body weight). Intestinal contents were removed by irrigation. Enterocytes were selectively removed by treatment with hyaluronidase 360 mg l^{-1} (Sigma

type V) followed by manual kneading of the intestinal loop as described by Peters and Shio (1976). The contents of the loop were removed, cells were spun down (2,000 x g for 15 min) and resuspended in PBS. An aliquot of cells was fixed in 3% glutaraldehyde, 0.1 M sodium cacodylate (pH 7.4) and 5% sucrose overnight at 20°C. After washing in cacodylate buffer, the fractions were postfixed in 1% osmium tetroxide, dehydrated, and embedded in Spurr resin. Ultra-thin sections were counterstained with uranyl acetate and lead citrate and then examined by electron microscopy. >70% of the cells were found to have morphological characteristics of enterocytes.

DNA Preparation

Human leukocyte DNA was prepared from whole blood by the Triton X-100 method of Kunkel et al. (1977). Liver or enterocytes were homogenized in buffer without sucrose and a nuclear pellet was collected by centrifugation through the same buffer containing 24% sucrose. The nuclear pellet was then digested with proteinase K in SDS as described by Kunkel et al. (1977).

RNA and Poly (A)$^+$ RNA Preparation

Total cellular RNA was prepared by the lithium chloride/urea method of Auffray and Rougeon (1980). Partially enriched poly (A)$^+$ RNA was produced by one or two cycles of binding to oligo (dT) cellulose (Aviv and Leder, 1972). The integrity of large and small apo-B mRNA was assessed by Northern blots.

Northern Blotting

Twenty micrograms of RNA was electrophoresed through 1% agarose gels containing 7% formaldehyde (Rave et al., 1979) and transferred to nitrocellulose. Prehybridization was at 42°C in 50% formamide, 5x SSC, 5x Denhardt's, 0.1% SDS, and 100 μg ml^{-1} denatured, sonicated salmon sperm DNA. Hybridization was for 16 hr in 10 ml fresh buffer containing 10^6 cpm ml^{-1} ^{32}P-labeled DNA. Filters were washed briefly at room temperature in 5x SSC/0.1% SDS then washed at 60°C in 1x SSC/0.1% SDS until the background was minimal. Autoradiography was on Kodak XAR5 film at −70°C with two Cronex intensifying screens for 24 hr.

cDNA Preparation

One to two micrograms of poly (A)$^+$ RNA (human terminal ileum or HepG2) or 30 μg of total RNA (rabbit liver and small intestine) were converted into double-stranded cDNA using a kit (Amersham PLC, cat. no. RPN 1256). Oligo (dT) or oligonucleotides PCR9 and PCR11 were used to make the first strand. First strand cDNA was converted to double-stranded cDNA according to Gubler and Hoffman (1983). On completion of double stranding, the products were purified by phenol:chloroform extraction followed by one ammonium acetate and one sodium acetate precipitation from ethanol. Transfer RNA was added as carrier when poly(A)$^+$ RNA was used to make cDNA. Yields were assessed in pilot reactions with α[^{32}P]dCTP label but the preparative reactions were performed without added isotope.

DNA Amplification

The polymerase chain reaction was carried out using Thermophylus aquaticus DNA polymerase under conditions recommended by the manufacturers (New England Biolabs). A cDNA (200 ng) or genomic DNA (1–3 μg) template was added to 67 mM Tris (pH 8.8) (at 25°C), 6.7 mM magnesium chloride, 16.6 mM ammonium sulphate, 10% DMSO, 10 mM β-mercaptoethanol, 6.7 μM EDTA, and 33 μM each dNTP in a 100 μl volume. The DNA was initially denatured at 96°C for 10 min followed by successive cycles of annealing at 40°C for 1 min, extension at 55°C for 5 min, and denaturation at 91°C for 1 min. Thermophylus aquaticus DNA polymerase (1–2 U) was added after the first denaturation step, and every tenth cycle thereafter until 30 cycles were completed.

Dot Blots

1:10 serial dilutions in H_2O of PCR reactions were dot-blotted onto Zetaprobe membrane using a BRL manifold. The membrane was washed in 0.4 M NaOH to fix the DNA and hybridized with the appropriate oligonucleotides.

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